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Bone Marrow Derived Cells do Not Engraft into Skeletal Muscle Microvasculature but Promote Angiogenesis Following Acute Injury

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Abstract

The skeletal muscle is supported by a vast network of microvessels with the capacity to regenerate in response to injury. However, the dynamics of microvascular repair and the origin of reconstituted endothelial cells in the skeletal muscle are poorly understood. A growing body of literature exists to indicate bone marrow (BM) derived cells engraft into regenerating vascular endothelium and muscle macro vasculature. Therefore we investigated the extent of BM contribution to skeletal muscle microvasculature following acute injury. Since reporters and markers commonly used to trace donor BM cells are not endothelial specific but also expressed by leukocytes, we generated novel BM chimeras utilizing *Tie2-GFP* BM cells transplanted into CD31 and Caveolin1 knockout recipients. In turn, we surveyed BM vascular contribution not just by the presence of GFP but also CD31 and Caveolin1 respectively. Following stable BM reconstitution, chimera limb muscles were cardiotoxin (CTX) injured and examined 21 days post injury for the presence of GFP, CD31 and Caveolin1. Acute muscle injury by CTX is characterized by initial microvasculature death followed by rapid endothelial regeneration within 14 days post damage. Histological analysis of injured and uninjured contralateral limb muscles, revealed a complete absence of BM engraftment in the muscle vasculature of wt and CD31/Caveolin1 KO chimeras. In contrast, F4/80+ cells isolated from CTX injured muscle, expressed endothelial related markers and promoted angiogenesis *in vitro*. Therefore, despite the absence of BM engraftment to regenerated skeletal muscle microvasculature, macrophages recruited following injury promote angiogenesis and in turn vascular regeneration.

Keywords

microvascular; skeletal muscle; endothelial; macrophage; bone marrow

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Disclosures

None

Supplemental Data and Methods

Detailed experimental methods and list of mouse strains and antibodies.

Introduction

For proper function and maintenance the skeletal muscle relies on an extensive vascular network. Damage to muscle vasculature by injury or disease can lead to ischemia which promotes debilitating and sometimes fatal conditions such as gangrene, non-healing wounds and peripheral artery disease. In contrast to muscle fiber regeneration, which has been extensively studied in acute injury models, regeneration of the skeletal muscle microvasculature has not been critically examined [1]. To date various cell populations including peripheral blood and bone marrow (BM) derived cells have been reported to promote angiogenesis and contributing to muscle vasculature [2–6]. However, many of the markers used to evaluate vascular contribution including Tie2 and CD31, are not exclusive to endothelial cells and can be expressed by circulating leukocytes, including monocytes [7–11]. Due to such overlap, putative vascular regeneration by BM derived populations is not easily distinguished from inflammatory cells responding to injury. Therefore, it remains unclear whether cells of BM origin actually contribute to regenerated endothelium or assist in the muscle repair process.

In this study we examined muscle vascular regeneration using the cardiotoxin (CTX) model of acute injury. CTX injury is characterized by initial massive tissue destruction followed by full regeneration within three weeks [12, 13]. To assess effects of CTX on muscle vasculature *in vivo*, we surveyed different time points following injury and observed an initial decline in skeletal muscle endothelial cells followed by regeneration to almost uninjured levels by day 14 post injury. BrdU and EdU incorporation indicates that endothelial cells, irrespective of their origin, proliferated in response to injury. To elucidate if vascular regeneration could be attributed to BM derived cells, we generated *Tie2-GFP* BM chimeras utilizing CD31 and Caveolin-1 knockout mice [14, 15]. Such models allowed us to effectively distinguish BM contribution into regenerating vasculature not just by the presence of the *Tie2*-promoter driven GFP, but also CD31 and Caveolin1 respectively [16]. Because impaired angiogenesis has been reported in both knockout models, we hypothesized these environments will favor *Tie2-GFP* BM contribution to vasculature following injury [14, 15, 17, 18]. Mobilization defects of BM endothelial progenitors has been reported in Caveolin1 deficient mice, adding to the selective advantage of normal BM in Caveolin1 knockout mice [19]. To exclude inflammatory monocytes responding to injury that may also express these markers, we examined chimeric animals 21 days following injury. Our results show the muscle endothelium was void of GFP in all chimeras and CD31 / Caveolin1 in each respective knockout model, indicating that following acute injury BM cells do not engraft in regenerated vasculature. In turn, as suggested by previous studies we demonstrate that macrophages recruited in response to muscle injury promote angiogenesis [20–22].

Methods

All mouse models used in this study were procured from Jackson labs and are detailed in the supplement. Animals were housed and maintained at the University of Washington in a modified barrier facility. Procedures used in this study were under the approval and guidance of the University of Washington's Institution Animal Care and Use Committee. Detailed experimental procedures and antibody specifics are included in the supplement.

Results

Following Acute Muscle Injury Endothelial Cells Decline but Quickly Regenerate

To investigate vascular regeneration in skeletal muscle we induced acute injury with CTX and examined endothelial decline by fluorescent-activated cell sorting (FACS) analysis in wt C57BL/6 mice [23]. In order to establish the kinetics of injury response we analyzed three

time points at day 3, 7 and 14 post injury as compared to undamaged Tibialis Anterior (TA) muscles (n=5 animals per time point). Only left TA's were injured and analyzed, while uninjured TA's from a separate group of mice were used as control for comparison. It has been demonstrated that endothelial cells are susceptible to CTX *in vitro* and the muscle capillaries density declines with CTX injury [24–26]. In accordance with these reports, we observed by FACS-analysis a rapid decline in the proportion of CD45⁺, Sca1⁺, CD31⁺ endothelial cells, 3 days post injury (figure 1A) [23]. TUNEL assay confirmed the initial endothelial cell decline observed 3 days post injury was due to cell death (figure 1B). Roughly 56% of CD31⁺ cells observed in fields of CTX injury were positive for nuclear localized TUNEL staining. In contrast to the initial decline, during the course of regeneration the proportion of endothelial cells increased at 7 days post injury and reached almost uninjured levels by day 14.

To assess if myogenic proliferation precedes endothelial cell regeneration, we conducted a BrdU pulse-chase experiment. CTX injury activates myogenic cells, with proliferation climaxing at day 3 post injury [13, 27]. Thus we chose to pulse animals (n=3 C57BL/6) for the first three days of injury and chase from day 4–14. We predicted that if endothelial cells and/or progenitors followed the same course of myogenic proliferation there would be a significant amount of BrdU incorporation and retention. Alternatively if endothelial cells proliferate following the peak of myogenic cell division beyond 3 days post injury, there would be low BrdU incorporation by day 14. Thus, low BrdU incorporation would indicate the vascular response is stimulated following the peak of myogenic cell proliferation. In contrast, results indicate proliferation occurred early after injury as endothelial cells retained a high degree of BrdU relative to bone marrow cells which continuously turnover and thus retained low amounts following the 10 day chase (figure 1C). Early proliferation indicated that resident surviving endothelial cells or circulating progenitors are activated and orchestrated with the myogenic response immediately after injury.

To further evaluate the course of vascular proliferation following injury, we examined by histology EdU incorporation in injured muscles from *Tie2-GFP* transgenic mice. Analogous to BrdU, EdU is incorporated into DNA during replication [16]. In contrast, EdU can be surveyed without acid or heat mediated DNA denaturization, which often leads to the loss of GFP and antigen recognition. To identify vascular cells we stained with Caveolin1 and examined EdU incorporation in muscles 3 and 7 days post CTX injury. Contralateral uninjured muscles were also surveyed for EdU incorporation. Staining revealed EdU⁺, Caveolin1⁺ cells in regenerating vasculature of injured muscles, confirming that vascular cells had proliferated directly following injury (figure 2). To our surprise, GFP fluorescence at day 3 and 7 post CTX injury was absent in Caveolin1⁺ cells suggesting the *Tie2-GFP* transgene is not active or expression is not sufficient for GFP to be visible above background fluorescence during the early stages of vascular regeneration. In contrast, *Tie2* promoter driven GFP was visible in vessels of uninjured contralateral muscles. However, we did not observe EdU incorporation in uninjured contralateral muscles. Therefore, vascular regeneration is not mediated by proliferating endothelial cells residing in distal muscles, but occurs from resident cells that survive injury or cells adjacent to the injured areas within the same muscle or adjacent muscles. Moreover, this data indicates damaged or dead skeletal muscle endothelium can repair itself following acute injury.

Chimera Knockout Models Are Effective Systems for Identifying BM Derived Vascular Contribution

High BrdU/EdU incorporation and retention during the first 3 days following injury suggested angiogenesis from regenerating damaged endothelium or neighboring undamaged vessels may be accompanied by alternate sources such as bone marrow (BM) derived progenitors. Since the majority of endothelial regeneration following CTX injury occurs

within 14 days, we hypothesized such rapid vascular reconstitution could not occur without contribution from cells of BM origin. To test our hypothesis we generated BM chimeras using *Tie2*-GFP donor BM. Muscle vasculature in this reporter model expresses *Tie2* promoter driven GFP and stain positive for CD31 and Caveolin1 (figure 3A). Because no single marker is exclusive to endothelial cells, *Tie2* being no exception, we generated BM chimeras in CD31 and Caveolin1 KO models (n=6 chimeras, 2× C57BL/6, 2× CD31 KO, 2× Caveolin1KO) (figure 3B). Such models allowed us to identify vascular contribution not only by the presence of GFP but also CD31 and Caveolin1 protein respectively. In addition, due to delayed angiogenesis in these knockout models, normal donor BM cells would incur a selective advantage over resident endothelial cells or progenitors [17, 18].

To assess BM vascular contribution in response to injury, we injected CTX in left limb muscles; TA, Quadriceps, and Gastrocnemius. Contralateral limb muscles were left uninjured to survey potential contribution in the absence of injury. Our characterization in the aforementioned results indicates CTX injury is ideal for studying endothelial regeneration as the majority of microvessels are massively damaged immediately after injury but rapidly repaired within 2 weeks. To distinguish definitive vascular contribution from vessel associated inflammation that occurs in response to damage, we chose to survey muscles following complete repair, 21 days post CTX injury (figure 3B). At this time point, wt muscles are fully regenerated with little or no inflammatory cells remaining. To quantify BM reconstitution we mismatched alloantigen donors and recipients. Conveniently we discovered that the FVB/N background *Tie2*-GFP reporter model harbors the strain specific CD45.1 alloantigen. Thus donor BM cells are easily distinguished from the CD45.2 expressed by C57BL/6 and related models including CD31 and Caveolin1 KO mice. FACS-analysis of BM mononuclear cells collected from each recipient at the end of the experiment indicated ~90% of all CD45+ cells were donor (CD45.1+) derived (figure 3C).

To date, a number studies have identified BM engraftment to vasculature via histological analysis [2, 3, 5, 6]. For our study we initially sought to utilize a combination of histological and FACS-analysis for identifying and quantifying BM derived endothelial cells. By histological analysis capillary, venous and arteriole endothelial cells in uninjured *Tie2*-GFP skeletal muscles, are all visibly positive for GFP. In order to assess the sensitivity and immunophenotype of GFP+ endothelial cells by flow cytometry, we conducted a validation experiment comparing *Tie2*-GFP and C57BL/6 limb muscles by FACS (figure 3D). Following our previously published FACS methods and characterization, we identified muscle endothelial cells as CD45⁻, CD31⁺ [23]. In turn the majority (95–97%) of endothelial cells analyzed from both *Tie2*-GFP and C57BL/6 limb muscles stained positive for Tie2. However, from *Tie2*-GFP muscles, GFP was not detectable in 38% of Tie2⁺ (CD45⁻, CD31⁺) endothelial cells. The discrepancy between Tie2 immunostaining and GFP may be attributed to the loss of GFP in cells whose membranes have been compromised by mechanical processing and enzymatic digestion. Such processing is commonly utilized and necessary for disassociating mononuclear cells from myofibers for FACS-analysis and/or cell sorting [28–30]. In turn, mononuclear cell isolation preserved antigen detection, but severely compromised the detection of cells that express GFP under the *Tie2* promoter making this approach impractical for detecting *Tie2*-GFP BM engraftment in skeletal muscle vasculature. Therefore, we chose to detect vascular engraftment by histological analysis for GFP and anti-GFP staining in order to ensure an accurate detection of *Tie2*-GFP + cells in BM chimeras.

BM Cells Did Not Engraft into Regenerated Muscle Vasculature

Following muscle regeneration 21 days post CTX injury, we harvested and froze injured and uninjured limb muscles (TA's, Gastrocnemius' and Quadriceps) for histological analysis. Initially we sectioned and surveyed transverse cross sections for the presence of GFP.

Relative to limb muscles processed the same day from *Tie2*-GFP mice, we did not detect any GFP signal in the vessels of BM chimeric animals (figure 4A). Few GFP+ cells were present only in regions with persistent inflammation in injured muscles of Caveolin1 and CD31 knockout mice. In contrast, C57BL/6 chimera muscles did not harbor significant inflammation 21 days post injury and were completely void of GFP positive cells. To confirm our findings we surveyed multiple cross sections (6 per muscle, spaced out ~200µm longitudinal distance) stained for CD31 and Caveolin1 from each BM recipient. Staining confirmed the absence of BM cell contribution to vasculature as indicated by the lack of CD31 and Caveolin1 immunoreactivity in the vessels of each respective KO chimera (figure 4A). Once more we only observed GFP+ inflammatory cells that stained positive for CD31 but negative for Caveolin1, in CD31 and Caveolin1 knockout mice (figure 4B). Negative vascular staining in each respective KO demonstrated antibody specificity. In contrast, the presence of antigen was validated by staining C57BL/6 control chimeras which showed robust signals for CD31 and Caveolin1 in vessels. Interestingly, analogous to our results in CTX injured muscles, uninjured contralateral muscles surveyed in parallel, were completely void of GFP and immunostaining for each respective KO protein. As a final test to confirm our finding we conducted anti-GFP staining. In line with the aforementioned results we did not observe GFP staining in the vessels of injured or uninjured chimeric muscles relative to control *Tie2*-GFP muscle (figure 5A). Once more, only in Caveolin1 and CD31 knockout chimeras where inflammatory cells persisted in injured muscles did we observe cells that stained positive for anti-GFP. These GFP+ cells also stained positive for VEGF receptor 2 (VEGFR2, aka Flk-1), but were not incorporated into vessels (figure 5B). Although BM derived endothelial progenitors have been reported to express VEGFR2, recent reports have shown macrophages and blood derived hematopoietic progenitors that do not give rise to endothelial cells also express VEGFR2 [6, 31, 32].

Although we did not observe GFP+ cells in vessels, we stained for CD45 to confirm the hematopoietic phenotype of *Tie2*-GFP+, VEGFR2+ cells observed in the injured muscles of CD31 and Caveolin1 knockout BM chimeras. Staining results demonstrate BM derived *Tie2*-GFP+ cells present only in CD31 and Caveolin1 knockout chimeras, are CD45+, Caveolin1- and localized near and sometimes adjacent to *Tie2*-GFP-, Caveolin1+ vascular cells (Figure 6). BM derived endothelial progenitor cells have been reported to express Caveolin1 but not CD45 [19, 31]. Therefore we inferred *Tie2*-GFP+ cells were remnant inflammatory cells and not bone marrow derived endothelial cells or progenitors, as they were positive for CD45 but negative for Caveolin1. In some cases, due to very close proximity, some Caveolin1+ vascular cells appear to overlap with CD45+ inflammatory cells. However the absence of Caveolin1 staining in Caveolin1 KO chimeras confirms *Tie2*-GFP+ and/or CD45+ inflammatory cells are indeed negative for Caveolin1. Therefore, we did not detect BM derived vascular incorporation in regenerated muscle microvasculature, but rather persistent inflammatory cells that express *Tie2*, *CD31* and *VEGFR2*, all markers associated with leukocytes and endothelial cells. Further characterization revealed that a *Tie2*-GFP+, CD45+ cells observed in knockout chimeras were also positive for F4/80, a marker of murine macrophages [33]. All F4/80+ cells were consistently positive for CD45, but not for GFP (Figure 6B). *Tie2*+ macrophages have been reported proangiogenic, and therefore the presence of F4/80+, *Tie2*-GFP+ cells indicates that persistent inflammatory cells remain to promote angiogenesis, which is perturbed in CD31 and Caveolin1 knockout chimeras [8, 10, 21, 22, 34, 35].

Macrophages responding to muscle injury promote angiogenesis

Recently it has been reported that a reduction of F4/80+ cells in muscle 3 days post CTX injury from CCR2 null mice, was associated with lower levels of VEGF and decreased capillary density during the course of regeneration [21]. Therefore, we hypothesized that

F4/80+ macrophages, which infiltrate muscle immediately following injury, directly promote angiogenesis. To investigate the angiogenic role of macrophages we isolated CD45+, F4/80+ cells 3 days post CTX injury for co-culture with skeletal muscle endothelial cells on matrigel and monitored vascular tube formation (Figure 7A and B). In order to account for potential vascular engraftment *in vitro*, CD45+, F4/80+ cells were isolated from animals that ubiquitously express GFP in all cells [36]. For comparison CD45+, F4/80- cells were also co-cultured and analyzed for endothelial related markers vs. CD45+, F4/80+ cells by FACS (Figure 7A). Interestingly, although a large proportion of CD45+ cells present in limb muscles 3 days post CTX injury were Tie2+ and Sca1+, few CD45+, F4/80- cells were positive for VEGFR2 and VE-Cadherin. In contrast ~30% of F4/80+ cells were positive for VEGFR2 and VE-Cadherin. Co-culture experiments revealed both FACS-sorted CD45+, F4/80+/- populations did not form vascular tubes, as indicated by absence of GFP in vascular tubes, but were closely associated with GFP- endothelial cells. However, only F4/80+ cells promoted angiogenesis while F4/80- cells appeared to impede vascular tube formation (Figure 7B).

To further investigate the role macrophages in promoting angiogenesis, we examined vascular branching from uninjured muscle explants plated on matrigel, in the presence or absence of F4/80+ cells, once more isolated 3 days post CTX injury. In contrast to aorta ring explants, skeletal muscle explants produce little vascular sprouting without robust pro-angiogenic intervention [37, 38] [39]. Therefore, muscle explants are ideal for evaluating the pro-angiogenic role of F4/80+ macrophages. Explants were cut from TA muscles of C57BL/6 and CD31 KO mice, to assay if macrophages can promote sprouting not only from wt but also from a model of perturbed angiogenesis [18]. Following 10 days of culture, vascular sprouting was significantly greater with F4/80+ cells in both wt and CD31 KO explants (Figure 7C). Altogether, such results indicate that macrophages responding to muscle injury directly promote angiogenesis. Furthermore, neither CD45+, F4/80+ macrophages nor CD45+, F4/80- cells formed individual vascular tubes or engrafted with co-cultured endothelial cell vascular tubes, supporting the absence of BM engraftment in muscle vasculature observed *in vivo*.

Discussion

Although vasculature contribution by BM derived cells has been reported for hindlimb ischemia injury, only single markers that are also expressed by leukocytes were examined[5]. BM derived endothelial progenitors not only share marker profiles with leukocytes but have also been reported indistinguishable from monocytes *in vitro* [11, 40, 41]. Therefore, legitimate endothelial contribution by BM derived cells is difficult to distinguish from inflammatory cells present after injury. Furthermore the engraftment of BM cells to tumor vasculature has been challenged, suggesting previous studies may have misinterpreted results by relying on single reporters such as *Tie2* that alone are not exclusive to endothelial cells [42]. Alternatively, differences in hind limb ischemia may be permissive to BM cell vascular contribution which varies from localized toxin injury that does not target the macrovasculature. With CTX injury, microvascular endothelial cell death and decline precedes massive vascular regeneration within 3 weeks of injury. In contrast, hindlimb ischemia injury models target the macrovasculature but has also been reported to result in muscle microvascular decline [43, 44]. Therefore, although hindlimb ischemia injury models present more obvious clinical relevance, CTX injury is a simple and technically straightforward model to study localized microvascular regeneration.

Herein this report, utilizing a novel system chimeras, we conclude that BM derived cells do not engraft with regenerating muscle microvasculature. In concordance with many reports, we observed that BM derived cells express common endothelial markers such as *Tie2*,

CD31, and *VEGFR*, but were not present in the regenerated vascular endothelium. Rather BM derived cells which stained negative for Caveolin1 were in close proximity to Caveolin1+ vascular cells and not clearly distinguished by single marker characterization. Although leukocytes play a significant role in injury repair, our data indicates an absence of BM-derived endothelial contribution to skeletal muscle vascular regeneration *in vivo* [22, 45]. Alternatively, BM contribution is transient and regresses once bona fide endothelial cells re-constitute damaged vasculature. Furthermore, we did not detect any BM derived cells in the vessels of uninjured muscles or with *in vitro* vascular tube formation, supporting the notion that BM contribution occurs in a transient manner under muscle duress or is injury model specific. Thus we conclude that if BM engraftment occurs, the event is temporary and not a permanent facet of vascular regeneration in skeletal muscle following toxin induced acute injury. In contrast, the presence macrophages in knockout chimeras, highlights the role of bone marrow cells in vascular regeneration. Subsequent experiments demonstrated that in contrast to CD45+, F4/80– cells present following injury, a greater proportion of macrophages (CD45+, F4/80+) were positive for endothelial related markers VEGFR2 and VE-Cadherin, and promoted vessel formation *in vitro*. Future research is warranted to elucidate the role(s) of macrophage/monocyte sub-populations in skeletal muscle vascular regeneration and in angiogenic impaired models such as CD31 and Caveolin1 knockout animals.

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Figure 1. Endothelial cell decline following acute injury is attributed to cell death

A) Time-course FACS-analysis of left Tibialis Anterior (TA) muscles (n=5 C57BL/6 per time point) following cardiotoxin (CTX) injury reveals the proportion of endothelial cells (EC) identified as CD45⁻, Sca1⁺, CD31⁺, initially declines but returns to near uninjured levels by day 14 post injury. TA's of uninjured animals were used for comparison. * indicates statistical significant between each group by single factor ANOVA, P<0.0005. Error bars = SEM.

B) Staining of injured C57BL/6 muscle, 3 days post CTX injury for cell death, reveals CD31⁺, TUNEL⁺ vessels (arrowheads). DNase1 treated muscle served as a positive control.

TUNEL staining in green corresponds to FITC and CD31 in red with AlexaFluor594. Scale bar = 50 μ m.

C) FACS-analysis of BrdU pulse-chase in CTX injured limb muscles (TA, Quadriceps, Gastrocnemius) indicates EC (CD45⁻, Sca1⁺, CD31⁺ sorted cells) retain a high levels of BrdU incorporated within the first 3 days post injury vs. bone marrow mononuclear cells (n=3 C57BL/6). As indicated by the gate, approximately 99% of all EC retained BrdU by day 14 post injury.

Figure 2. Vascular regeneration begins directly following muscle injury

A) Staining for EdU incorporation and vascular cells with anti-Caveolin1 at 3 days and 7 days post CTX injury in muscles from *Tie2-GFP* reporter animals, reveals vascular cells proliferated directly following injury to replace the damaged endothelium. Arrows point to EdU+, Caveolin+ cells within injured areas of muscle. Surprisingly, GFP was not readily visible suggesting the transgene is not expressed during the early stages of vascular regeneration and remodeling.

B) Staining for EdU in uninjured contralateral muscles indicates an absence of incorporation. However, *Tie2* promoter driven GFP was visible in the undamaged endothelium. Scale bars = 50 μ m.

Figure 3. *Tie2*-GFP reporter animals express GFP in skeletal muscle vasculature and harbor the CD45.1 alloantigen, making them ideal for chimera generation

A) Analysis of muscle cross-sections reveals *Tie2* promoter driven GFP is expressed by muscle endothelial cells and co-localizes with CD31 and Caveolin1 staining. Scale bar = 50 μ m.

B) Experimental schematic of allogeneic chimera generation using *Tie2*-GFP bone marrow into donors and C57BL/6, CD31 and Caveolin1 knockout mice, and subsequent injury.

C) FACS-analysis of chimera limb derived bone marrow mononuclear cells reveals the average reconstitution of donor BM based on the percentage of CD45+ cells was ~90% between all chimeras analyzed (n=6). C57BL/6 BM cells express the CD45.2 alloantigen

(left graph). Conveniently we discovered that *Tie2-GFP* animals on the FVB/N background express the CD45.1 (middle graph). Thus donor cells in BM chimeras are easily distinguishable from recipients which express CD45.2 (right graph).

D) FACS analysis of endothelial cells from *Tie2-GFP* limb muscles indicates detecting of GFP by flow cytometry following enzymatic muscle digestion is compromised. As compared to the unstained control, 95–97% of endothelial cells identified as CD45⁺, CD31⁺ in *Tie2-GFP* and C57BL/6 muscles, stained positive for Tie2. In contrast, GFP was detected in only 57% of Tie2⁺ endothelial cells. Therefore, FACS is not an accurate method for detecting *Tie2-GFP*⁺ endothelial cells in skeletal muscles of BM chimeras.

Figure 4. Absence of bone marrow vascular contribution following acute injury

A) Histological analysis revealed an absence of GFP in the regenerated muscle vasculature of all chimeric animals. Staining for CD31 and Caveolin1 confirm the absence of BM derived cells in the endothelium of larger vessels (arrow) and capillaries (arrowhead pointing to one of many) of injured muscles (Quadriceps shown) of knockout animals. Vessels in uninjured contralateral muscles were also negative for GFP and CD31/Caveolin1 in each respective knockout (not shown).

B) GFP+ cells were only observed in regions of persistent inflammation only in CD31 and Caveolin1 knockout mice (CD31 KO shown). *Tie2-GFP*+ BM derived inflammatory cells were positive for CD31 but negative for Caveolin1 (arrowhead). Scale bars = 50µm.

Figure 5. Bone marrow derived cells were present only in areas with persistent inflammation in the injured muscles of knockout chimeras

A) Staining for anti-GFP confirmed a complete absence of bone marrow cells in regenerated muscles of C57BL/6 chimeras. GFP was detected only in areas with persistent inflammation in the injured muscles of CD31 and Caveolin1 knockout animals (not shown here). *Tie2-GFP* muscle processed in parallel with chimeras indicates GFP was preserved and co-localized with antibody staining.

B) Bone marrow derived *Tie2-GFP*⁺ cells (arrowheads) detected with anti-GFP in knockout chimeras (CD31 KO shown), stained positive for VEGFR2 and were adjacent to VEGFR2⁺ vessels (arrows). Inset (top left of merge) highlights the proximity of VEGFR2⁺ vessels and anti-GFP⁺, VEGFR2⁺ cells. Scale bar = 50μm.

Figure 6. *Tie2-GFP*+ bone marrow cells represent inflammatory cells that do not engraft to regenerated muscle microvasculature

A) *Tie2-GFP*+ cells (arrowheads) observed only in CD31 and Caveolin1 knockout chimeras are remnant or persistent inflammatory cells that stain positive for CD45 but negative for Caveolin1. The absence of Caveolin1 staining in Caveolin1 KO chimeras confirms that only vascular cells but not *Tie2-GFP*+ and/or CD45+ inflammatory cells are positive for Caveolin1. The observed close proximity of *Tie2-GFP*+ BM derived cells to Caveolin1+ vascular cells may obscure the identification of engraftment from vessel associated inflammation, when only one marker or reporter common to both cell types is used. Scale bar = 50µm.

B) Staining for the macrophage marker F4/80 reveals a proportion of F4/80+ cells express *Tie2-GFP* (arrowheads). Although all F4/80+ cells were CD45+, not all were positive for GFP (arrow). Scale bar = 50µm.

Figure 7. F4/80+ macrophages recruited following muscle injury promote skeletal muscle microvascular angiogenesis *in vitro*

A) FACS analysis and sorting schematic from a pool of limb muscles 3 days post CTX injury, the time point preceding vascular regeneration. CD45+ cells from injured muscles were selected and sorted as F4/80+ and F4/80- for co-culture experiments. An IgG isotype control conjugated to PE was used to confirm the absence of non-specific staining. Aliquots taken from the same preparation labeled with CD45 and F4/80, were stained with PE conjugated antibodies against Tie2, VEGFR2, VE-Cadherin, and Sca1 to analyze the presence of each respective antigen on CD45+, F4/80+ (middle row of histograms) and CD45+, F4/80- cells (bottom row of histograms). Histogram red peaks represent the

unstained control, blue peaks represent stained experimental samples. Percentages represent the proportion of event within gates (horizontal black bars), for each respective population. B) Co-culture of skeletal muscle microvascular endothelial cells with GFP expressing CD45⁺, F4/80⁻ or F4/80⁺ cells, reveals only F4/80⁺ cells (macrophages) promote vascular tube formation on matrigel. Although neither population engrafted with vascular tubes, GFP⁺ cells were observed in close proximity to endothelial cells. Scale bar = 50 μ m. C) CD45⁺, F4/80⁺ cells FACS-sorted 3 days post CTX injury, promote vascular sprouting from both uninjured C57BL/6 and CD31 KO Tibialis Anterior muscle explants (n=3 explants per condition) seeded on matrigel. Scale bar = 100 μ m * $P \leq 0.05$ by two-tailed Student's *t*-test.